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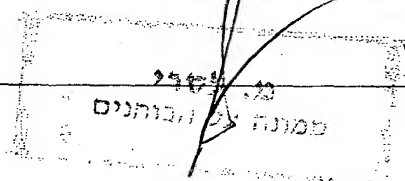
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בקשה לפטנט
Application For Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגדת מקום התאגדותו)
I, (Name and address of applicant, and in case of body corporate-place of incorporation)

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(בעברית)
(Hebrew)

Cyclic glycerophosphates and analogs thereof

(באנגלית)
(English)

Hereby apply for a patent to be granted to me in respect thereof.

מבקש בזאת כי ינתן לי עליה פטנט

בקשת חלוקה Application of Division		בקשת פטנט מוסף Appl. for Patent of Addition		דרישת דין קדימה Priority Claim		
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Cyclic glycerophosphates and analogs thereof

ידע חברה למחקר ופיתוח בע"מ

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C. 115444

CYCLIC GLYCEROPHOSPHATES AND ANALOGS THEREOF

FIELD OF THE INVENTION

The present invention concerns cyclic glycerophosphates and analogs thereof.

5 PRIOR ART

The following is a list of references which is intended for a better understanding of the background of the present invention.

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- Tomac, A., *et al.*, *Nature*, **373**:335-339 (1995).
- 10 Ukita, T., Bates, N.A. and Carter, H.E., *J. Biol. Chem.*, **216**:867-874, (1955).

BACKGROUND OF THE INVENTION

L- α -glycerophosphate (α GP), a key constituent in phospholipid metabolism (Kennedy and Weiss, 1956), is abundant in most biological tissues
15 (Dawson, 1958). β -Glycerophosphate (β GP) is a product of enzymatic (Ukita *et al.*, 1955) and alkaline (Clarke and Dawson, 1976) hydrolysis of phospholipids and is formed through the cyclic phosphodiester intermediate 1,2-cyclic glycerophosphate (1,2 cGP) (Ukita *et al.*, 1955; Clarke and Dawson, 1976). 1,2 cGP has been detected in algae species (Boyd *et al.*, 1987) as well as
20 in human cancer tissues (Su *et al.*, 1993). Similarly, α GP can in principle adopt the cyclic form 1,3-cyclic glycerophosphate (1,3 cGP). This compound has been shown to be formed as an intermediate in the phospholipase C hydrolysis of phosphatidyl glycerol (PG) (Shinitzky *et al.*, 1993) and upon further hydrolysis is converted to α GP.

25 A six-membered cyclic phosphate of foremost biological importance is cyclic AMP. The ring of cyclic AMP is actually a derivative of 1,3 cGP backbone. Other cyclic phosphates which were detected in biological systems include glucose cyclic phosphodiester (Leloir, 1951), 2',3'-cyclic phosphodiester (Markham and Smith, 1952), riboflavin-4',5'-cyclic
30 phosphodiester (Forrest and Todd, 1950), myoinositol-1,2-cyclic phosphodiester (Dawson *et al.*, 1971) and cyclic lysophosphatidic acid (Friedman *et al.*, 1996).

Except for cyclic AMP and cyclic GMP which have been extensively studied, no specific biological activities have been so far assigned to the other biological cyclic phosphates.

5 **List of compounds and their abbreviations**

The following compounds which formulas are presented in **Appendix A** just before the claims, will be represented herein in the specification by their abbreviations as follows:

1. 1,3 cyclic glycerophosphate - **1,3 cGP**
- 10 2. 1,2 cyclic glycerophosphate - **1,2 cGP**
3. 3-acyl 1,2 cyclic glycerophosphate (cyclic lysophosphatidic acid) -
 c-lysoPA
4. Phenyl 1,3 cGP - **P-1,3 cGP**
5. Phenyl 1,2 cGP - **P-1,2 cGP**
- 15 6. 1,3 cyclic propanediol phosphate - **1,3 cPP**
7. 1,2 cyclic propanediol phosphate - **1,2 cPP**
8. Phenyl 1,3 cPP - **P-1,3 cPP**
9. Phenyl 1,2, cyclic propanediol phosphate - **P-1,2, cPP**
10. Cyclic dihydroxyacetone phosphate - **cDHAP**
- 20 11. Phenyl cyclic dihydroxyacetone phosphate - **P-cDHAP**

GLOSSARY

The following is an explanation of some terms used above and in the following description and claims:

25

Target cells - cells in which, following contact with the GCs of the invention, there is phosphorylation of intracellular proteins. In some cases, contact of the target cells with GCs results in maturation of the cells and in other cases in

hormone-like signaling activities. In addition, a variety of cellular events may occur in the target cells following their contact with the GCs of the invention.

Intracellular phosphorylation (used interchangeably with phosphorylation of
5 intracellular proteins) – rise in the level of phosphorylation in one or more of the
intracellular proteins of the target cells following incubation of the cells with the
GCs of the invention. The phosphorylation is typically of the tyrosine amino acid
in the protein but may also be of the threonine or serine amino acid. The protein
may be any protein inside the target cell that can be phosphorylated. Typically the
10 protein in which phosphorylation occurs is constitutively phosphorylated to some
extent and the level of its phosphorylation is effected by the GCs. The level of
phosphorylation may be determined using any of the methods known in the art
such as those described below.

15 *Promotion of cell differentiation* – the activity of the GCs of the invention
causing changes in the target cells which are correlated with the differentiation
stage of the cells. The changes may be in anatomical characteristics, in the
expression of differentiation antigens, etc.

20 *Induction of hormone-like signaling* - the activity of the GCs of the invention
on target cells which results in changes which are typically induced by hormones.
The GCs applied externally to the target cells pass through the cell membrane and
exert their effect inside the target cells. For example, in target cells expressing the
insulin receptor, such changes may be similar to the effects exerted by insulin on
25 the same cells.

Analog - relates to any compound which is derived from one of the cyclic
glycerophosphates of the invention and which substantially maintains the
activity of the cyclic phosphate from which it was derived, including, for

example, deoxy analogs and phenyl esters of the cyclic phosphates, preferably, deoxy analogs.

Substantially maintaining - this term relates to the analogs ability to promote the activity carried out by the compound from which they were derived to a certain extent. The analog's activity will be considered to be substantially maintained wherein the activity is 30% or above, preferably 50% or above, more preferred 70% or above, and most preferably 90% or above the level of the activity of the compound.

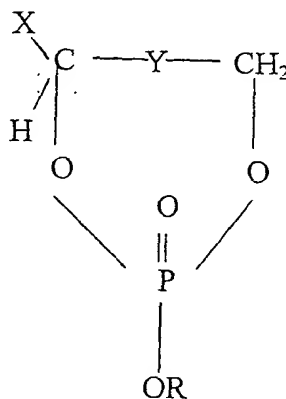
Effective amount - wherein the method of the invention is intended for prevention of a non-desired condition, the term "*effective amount*" should then be understood as meaning an amount of the active compound which, when administered, results in the prevention of the appearance of the said condition. Prevention of such a condition may be required prior to the appearance of any symptoms of a disease, e.g. in individuals having a high disposition of developing the disease. Wherein the compositions or methods are intended for treatment of an ongoing non-desired condition, the term "*therapeutically effective amount*" should then be understood as meaning an amount of the active compound which is effective in ameliorating or preventing the enhancement of the treated condition and related symptoms, which reduces the undesired symptoms or which completely eliminates them. In accordance with the "*in vitro*" aspect, the term "*effective amount*" relates to an amount of the active material which, upon contact with the target cells, causes the desired change in said cells.

SUMMARY OF THE INVENTION

It has now been found, in accordance with the present invention, that extracellular application of cyclic glycerophosphates and analogs to target cells

increases within minutes the level of phosphorylation in intracellular proteins of the cells. Linear α GP and linear β GP on the other hand, lack this activity.

The present invention thus provides, by a first of its aspects, a pharmaceutical composition comprising a pharmaceutically acceptable carrier and, as an active material, a compound of the general formula I:



wherein

Y is $-(\text{CH}_2)_m-$, $-\text{CH}(\text{OH})-$ or $-\text{C}(=\text{O})-$, and m is 0 - 3 ;

X is H, alkyl, $-\text{CH}_2\text{OH}-$, CH_2Oacyl or $-\text{CH}_2\text{acyl}$; and

R is H, a cation, alkyl or optionally substituted aryl;

or an analog of said compound.

As used herein the term "*alkyl*" refers to an alkyl group having from about 1 to about 24 carbon atoms, e.g. preferably from about 3 carbon atoms to about 20 carbon atoms, most preferably from about 5 carbon atoms to about 15 carbon atoms; the term "*acyl*" refers to an aliphatic saturated or unsaturated $\text{C}_1 - \text{C}_{24}$ acyl group, preferably an acyl group having an even number of carbon atoms, most preferably an acyl group derived from a natural fatty acid such as a saturated aliphatic acyl group selected from acetyl, butyryl, caproyl, octanoyl, decanoyl, lauroyl, myristyl, palmitoyl and stearoyl, or an unsaturated aliphatic acyl group selected from palmitoleyl, oleyl, linoleyl, and ricinoleyl; and the term "*aryl*" refers to a mono- or poly-carbocyclic aryl group, most preferably phenyl, optionally substituted by $\text{C}_1 - \text{C}_4$ alkyl, halogen and/or hydroxy.

Where m in the above formula I is 0, X is preferably $C_1 - C_{24}$ alkyl, most preferably methyl. By a preferred embodiment, the above composition comprises 1,2-cyclic propanediol phosphate or 1,3-cyclic propanediol.

By an additional embodiment m in formula I is 0 and X is CH_2OH . In accordance with this embodiment, preferably the composition comprises 1,2-cyclic glycerophosphate or phenyl 1,2-cyclic glycerophosphate.

By yet another embodiment, where m in formula I is 0, X is CH_2acyl and the composition preferably comprises 3-acyl-1,2-cyclic glycerophosphate, most preferably cyclic oleyl lysophosphatidic acid.

Where Y in the above formula I is $-CH_2-$, preferably the composition comprises 1,3-cyclic propanediol phosphate or phenyl 1,3-cyclic propanediol phosphate.

Where Y in the above formula I is $-CH(OH)-$ the composition preferably comprises 1,3-cyclic glycerophosphate or phenyl 1,3-cyclic glycerophosphate.

Where Y in the above formula I is $-C(=O)-$ the composition preferably comprises cyclic dihydroxyacetone phosphate or phenyl cyclic dihydroxyacetone phosphate.

Wherein R in the above formula I is a cation, it is preferably Na.

The compounds of the above Formula I are novel with the exception of the following compounds: (i) compounds wherein Y is $-(CH_2)_m-$, m is 0, X is CH_3 , $-CH_2OH$ or CH_2Oacyl wherein acyl is a saturated carboxylic acyl with more than 12 carbon atoms, and R is H or a cation; (ii) compounds wherein Y is $-(CH_2)_m-$, m is 1, X is H and R is H, a cation or phenyl; and (iii) compounds wherein Y is $-CH(OH)-$, X is H and R is H, a cation or phenyl.

Examples of the new compounds are the compounds:

phenyl 1,2 cyclic glycerophosphate

phenyl 1,2 cyclic propanediol phosphate

cyclic dihydroxyacetone phosphate

phenyl cyclic dihydroxyacetone phosphate
cyclic oleyl lysophosphatidic acid.

By another of its aspects, the present invention provides a pharmaceutical composition for inducing phosphorylation in intracellular
5 proteins of target cells comprising a pharmaceutically acceptable carrier and, as an active material, a compound of general Formula I above.

Such phosphorylation of proteins is known to be an essential stage of many signaling pathways which are involved in cellular processes. The phosphorylating activity of the cyclic glycerophosphates of the invention
10 renders them as useful in the prevention and treatment of various disorders and diseases.

The present invention also provides a pharmaceutical composition for treatment of disorders and diseases which can be treated by phosphorylation of internal proteins comprising a pharmaceutically acceptable carrier and, as an
15 active material, a compound of general Formula I above.

In addition, the present invention provides a method for treatment of disorders and diseases which can be treated by phosphorylation of internal proteins comprising administering to the individual in need a therapeutically effective amount of a compound of general Formula I above.

20 One cellular process which involves phosphorylation in intracellular proteins is differentiation. The present invention also provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and, as an active material, a compound of the general Formula I above for promotion of cell differentiation in target cells.

25 The capability of the compositions of the invention to induce cell differentiation in target cells makes them especially suitable for use in the treatment of various disorders and diseases such as various malignancies. Examples of such diseases are blood malignancies such as leukemias and lymphomas, solid tumors such as breast cancer or brain tumors, etc.

By an additional aspect, the present invention also provides a method for the treatment of malignant diseases comprising administering to an individual in need a therapeutically effective amount of the compound of Formula I above.

In addition, due to the capability of the compositions of the invention to
5 induce hormone-like signaling, they may be used for the prevention or treatment of various disorders in which such hormone signaling is involved.

By yet another of its aspects, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and, as an active material, a compound of the general Formula I above for
10 induction of hormone-like signaling.

The hormone-like signaling activity of the pharmaceutical composition of the invention may effect the target cell in a similar manner as that of the typical hormone which effects the cells and/or may be synergistic with the activity of the hormone resulting in an elevated signal in the treated cells. In
15 accordance with this aspect of the invention, the GCs may, for example, be used for induction of an insulin-like signal. This may be useful in the treatment of non-insulin dependent diabetes mellitus (IDDM) - type II diabetes. In addition the GCs may be used for induction of the signal of human growth hormone (HGH) in the treatment of disorders in which HGH is involved, for
20 induction of epidermal growth factor (EGF) for use in the treatment of disorders involving EGF, etc.

By yet another of its aspects, the present invention provides a method for the treatment of diseases involving hormone-like signaling comprising
~~administering to an individual in need a therapeutically effective amount of the~~
25 compound of the Formula I above.

~~In addition, the compounds of the invention may be used to prepare~~
medicaments suitable for treating diseases and disorders such as those mentioned above.

Being involved in crucial signaling pathway of the cell, the level of phosphorylation of intracellular proteins may be used as an indicator of certain cellular situations. For example, there may be disorders which effect the activity of kinase enzymes or phosphatase enzymes resulting in abnormal levels of phosphorylation in response to extracellular stimulations. By contacting cells of interest with the GCs of the invention, it is possible to measure the level of phosphorylation in the cells and to compare it to the level of phosphorylation in normal cells. A level of phosphorylation which differs from that in normal cells may indicate an abnormal condition in the tested cells. Thus, the present invention also provides a method for detecting abnormal conditions of a tested cell comprising:

- i. contacting the cells with glycerophosphates;
- ii. detecting the level of phosphorylation in intracellular proteins of the tested cells; and
- 15 iii. comparing said level of phosphorylation to the level of phosphorylation in intracellular proteins of normal cells following their contact with said GPs, a level of phosphorylation differing from that detected in the normal cells indicating a high probability of abnormality in the tested cells.

20

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention it has been found that cyclic glycerophosphates (GCs) and some of their analogs are involved in signaling pathways in cells. ~~Linear α and β glycerophosphates do not exert such an activity.~~

25

~~Cyclic glycerophosphates can be formed by enzymatic degradation of phospholipids which in most cases yields five or six membered ring cyclic phosphates.~~ The present invention encompasses within its scope both such cyclic glycerophosphates formed by enzymatic degradation of phospholipids as well as

synthetically formed ones. CGs having rings of less than five or more than six carbon atoms are also included within its scope.

The cyclic phosphates of the invention may generally be synthesized using any one of the methods known in the art for synthesis of phosphate esters. Specific methods which may typically be used for preparing the cyclic phosphates
5 of the invention are described specifically below (see Examples).

Analogous of these cyclic glycerophosphates of the invention are also within the scope of the invention being typically deoxy analogs as well as phenyl esters of the 1,3 cyclic phosphates. These analogs may also be prepared by enzymatic
10 methods or synthetically by any of the methods known in the art.

In addition to the active material, the pharmaceutical compositions of the invention may also contain a carrier selected from any one of the carriers known in the art. The nature of the carrier will depend on the intended form of administration and indication for which the composition is used. Examples of
15 such carriers are saline, pills or capsules, liposome suspensions, etc. The compositions may also comprise a number of additional ingredients such as diluents, lubricants, binders, preservatives, etc.

The signaling activity of the CGs of the invention is exerted through their capability to induce phosphorylation of intracellular proteins in cells. When the
20 CGs are applied onto the cells, a rapid phosphorylation is observed in intracellular proteins. Typically, the phosphorylating activity of the GCs may be observed after a period of between about 0.5 mins. to about 20 mins. of contact with the target cells.

~~In accordance with the invention, the phosphorylating activity of the GCs~~
25 ~~may be measured by any of the methods known in the art. Generally, following~~
~~incubation of the GC with the target cells, the cells are lysed, the protein~~
concentration in each sample is determined and the level of phosphorylation of the proteins is determined using known methods and suitable and available polyclonal or monoclonal antibodies. Typically, phosphorylation occurs on the

tyrosine residues of the protein and such phosphorylation is determined using either polyclonal or monoclonal anti phosphotyrosine antibodies (such as those described above). However, in some cases, phosphorylation occurs on the threonine or serine components of the proteins in which case polyclonal or
5 monoclonal anti phosphothreonine or anti phosphoserine antibodies may be used.

The molecular weight of the proteins in which phosphorylation occurs following incubation with various kinds of CGs may also vary to a certain extent. Thus, for example, incubation of CHO and NIH-3T3 cells with six membered ring cyclic phosphates resulted in phosphorylation of proteins having a molecular
10 weight of about 35 kD, about 45 kD, about 60 – 70 kD and about 120 kD. The molecular weight of the proteins found to be phosphorylated following incubation of the same cells with five membered ring cyclic phosphates was about 18 kD, about 35 kD and about 38 kD.

The cPGs of the invention may be administered to cells *in vitro*. Such
15 administration may result in desired changes in the cells which may then be administered back to an individual in need. In addition, administration of the compositions to cells may result in enhanced secretion of various growth factors by these cells which, in turn, could also be used for treatment of various conditions alone or in combination with other cPGs of the invention.

20 The effective amount of the GC to be used *in vitro* in accordance with the invention may vary in accordance with the nature of the CG as well as the target cells and can easily be determined by a person versed in the art by using any of the above-mentioned methods or any of the methods known in the art. For *in vitro* induction, the typical range of concentration of CG needed to induce
25 phosphorylation is between about 0.5 μ M to about 10 μ M.

~~Many cellular processes are triggered by protein phosphorylation (mostly~~
tyrosine phosphorylation). Thus, application of the GCs to target cells results in various cellular processes. One such process, is cell differentiation. Such differentiation may easily be determined by a person skilled in the art by

measurement of parameters and characteristics in the target cells upon their differentiation. The CGs capability of inducing differentiation of cells makes them useful in the prevention or treatment of disorders or diseases in which differentiation of cells is desired such as, for example, in various malignant diseases.

The GCs of the invention are also able to exert hormone-like signaling activities in target cells. Thus, for example, the GCs of the invention may exert insulin-like activity on cells expressing the insulin receptor. This makes the GCs of the invention suitable candidates for treatment of disorders or diseases involving hormone signaling. The GCs of the invention may also be used in synergism with known hormones, e.g. together with insulin in the treatment of IDDM.

Where the compositions of the invention are administered *in vivo*, a preferred mode of their administration is either i.v., topically or per os although at times it may be advantageous to use other administration modes as well.

Typically, the pharmaceutical compositions of the invention will comprise about 1 mg to about 10 mg of the active material per kg body weight of the treated individual.

While the compositions of the invention will typically contain a single cGP, it is possible at times to include in the composition or to co-administer two or more cGPs which may then act together in a synergistic or additive manner to prevent or treat the specific disorder.

According to the invention, the cPGs may be administered either in a single dose or may be given repetitively over a period of time.

The compositions of the invention may also be administered to the treated individual in combination with an additional treatment, e.g. wherein the treated condition is a malignant one, the compositions may be given together with one of the currently available drugs or therapies used for treatment of such diseases such as various chemotherapeutic drugs together with a growth factor such as

Interleukin-2 (IL-2). In another example, the CGs of the invention may be administered to an individual suffering from IDDM in combination with insulin. In such a combination treatment the cPGs may be administered simultaneously with or at different times than the administration of the additional treatment so as to yield a maximum preventive or therapeutic effect.

The induced increase in intracellular phosphorylation by cyclic phosphates of the invention may be the result of the effect of the cyclic phosphates on one of several routes including activation of intracellular kinases (e.g. MAPK) on the one hand and inhibition of phosphatase activity on the other hand. Each of the above routes which may occur separately or in combination results in the augmentation of the degree of phosphorylation of intracellular proteins. The apparent degree of phosphorylation of such proteins is, most likely, at a steady state between counteracting kinase and phosphatase activities.

Without being bound by theory, and on the basis of the results of the present invention, the GCs of the invention may exert their activity in the following way: upon application of the cyclic phosphate to the target cell, the cyclic GC first partitions into the cytosol of the cell and when it reaches certain local concentration (of between about 0.1 to about 1 μ M) is capable of activating kinases in the cytosol (such as MAPK). At the same time the GCs also inhibit phosphatase activity. The activation of kinases and inhibition of phosphatase activity results in induction of phosphorylation of tyrosine or serine/threonine phosphorylations in a series of proteins. As the concentration of the GCs in the cytosol rises, phosphodiester activity cleaves the active cyclic phosphates reducing their above activities and resulting in a reduction in the phosphorylation level of proteins in the cells.

The cyclic phosphates of the invention may be used in any of their isomer forms. For various purposes, one of the isomers may be preferred over the remaining ones. For example, amongst the four stereo isomers which

constitute the synthetic 1,3 cGP depicted in Appendix A, the enzymatic product 1,3 cGP(E) is preferred for use for inhibiting the overall intracellular phosphatase activity.

5 **EXAMPLES**

The invention will now be illustrated by the following non-limiting examples with reference to the appended figures.

Fig. 1 shows the level of tyrosine phosphorylated proteins in CHO cells after 1 min. pulsing at 37°C with 1 μ M α GP, β GP, 1,3 cGP (E), P-1,3 cPP and 1,3 cPP. Detection with polyclonal anti-phosphotyrosine antibodies.

Fig. 2 shows the level of tyrosine phosphorylated proteins in CHO cells after 1 min pulsing at 37°C 1 μ M and 2 μ M 1,3 cPP. Detection with monoclonal anti-phosphotyrosine antibodies.

Fig. 3 shows the level of tyrosine phosphorylated proteins in NIH 3T3 cells after 1 min pulsing at 37°C with 1,3 cPP. Detection with monoclonal anti-phosphotyrosine antibodies.

Fig. 4 shows the time course of tyrosine phosphorylation in proteins of CHO cells pulsed with 5 μ M and 10 μ M 1,2 cPP for a period of 1 min., 3 mins. or 5 mins. . Detection with monoclonal anti-phosphotyrosine antibodies.

Fig. 5 shows the ³²P labelled proteins of CHO cells after 2 min pulsing with 2 μ M 1,3 cGP (E) or 1,3 cPP followed by gel electrophoresis and in-gel treatment with 1 M KOH.

Fig. 6 shows the in-gel assay for kinase identification of proteins from CHO cells phosphorylated by 2 μ M 1,3 GP or 1,3 cPP in comparison to 2 μ M α GP or β GP (control). In-gel identification of Raf-1, MAPK and Jun kinase in the phosphorylated proteins is shown in the lower panel.

Fig. 7 shows the time profile of threonine phosphorylation in CHO cells induced by 4 μ M 1,3 cGP (E) at 25°C. Abolishment of detection of threonine

phosphorylation (a) by the presence 5 mM phosphoserine (b) or 5 mM phosphothreonine (c) is also shown.

Fig. 8 shows the uptake profile of 1,3 cP³²P (10 μM) by CHO cells. The inserts presents the magnified profile for short incubation times.

5 Fig. 9 shows the displacement profiles of 1,3 cP³²P in perforated CHO cells by αGP (x-x), unlabelled 1,3 cPP (filled symbols, 3 separate experiments) and 1,3 cGP (E) (open symbols, 2 separate experiments).

Fig. 10 is a schematic representation showing the relative level of phosphorylation of insulin receptors on CHO-T cells following their incubation with insulin (1 nM or 0.1 nM), 1,3, cPP (0.1 μM) or a combination of both. Fig. 10A shows phosphorylation of the 117 kD band of the insulin receptor and Fig. 10B shows phosphorylation of the 200 kD band of the insulin receptor.

CHEMICAL SECTION

15 Synthesis of the cyclic phosphates

The cyclic phosphates of the invention are prepared by the reaction of a suitable dihydroxy compound wherein Y is - (CH₂)_m - or - C(=O) - and X is H or alkyl with phosphorus oxychloride (POCl₃) when R is H or with aryl, e.g. phenyl, phosphorodichloridate (RO-P(=O)Cl₂) when R is aryl.

20 When there is one or more hydroxy groups in the starting compound, namely Y is - CH(OH) - and/or X is - CH₂OH -, these hydroxy groups have to be protected, e.g. by benzylation, and the benzyl group is then removed after cyclization by conventional catalytic hydrogenation in the presence of a suitable catalyst such as Pt or Pd.

25 The reaction is carried out in an anhydrous solvent, e.g. dioxane or methylene chloride, in the presence of equivalent amounts of a nucleophile such as pyridine or triethylamine. The end products, when R is not aryl, are usually obtained as salts.

The synthesis of a series of known and novel 5- and 6-membered ring cyclic phosphates is illustrated below.

Example 1: Synthesis of 1,3 cyclic glycerophosphate (1,3 cGP) and 1,3,cGP(E)

The procedure of Buchnea (Buchnea, 1973) was followed essentially as described. Briefly, 2-benzyloxy-1,3-propanediol (Aldrich) was reacted with an equimolar amount of phosphorus oxychloride (Aldrich) in methylene chloride. The resulting 2-benzyl-1,3 cGP was treated with hydrogen under the catalysis of Pd black in methanol to remove the benzyl residue. The 1,3 cGP, isolated as the Ba salt, was pure on paper chromatography (n-propanol: ammonia: water 6:3:1, $R_f=0.52$).

1,3 cGP was also produced by the cleavage of phosphatidyl glycerol (PG) with phospholipase C as described (Shinitzky et al., 1993). The product, termed 1,3,cGP(E) had a trace of approx. 10-20% α -GP as indicated by paper chromatography.

Example 2: Synthesis of 1,2 cyclic glycerophosphate (1,2 cGP)

This compound was prepared as described (Kugel and Halmann, 1967). The disodium salt of β -glycerophosphate (Sigma) was first converted to the acid form and then cyclized with dicyclohexylcarbodiimide (Aldrich). The product, isolated as the Ba salt, was pure on paper chromatography.

Example 3: Synthesis of phenyl 1,3 cyclic glycerophosphate (P-1,3 cGP)

The method described in Example 1 for 1,3 cGP was followed by reacting 2-benzyloxy-1,3-propanediol with phenyl phosphorodichloridate (Aldrich). The intermediate benzylated product was pure on thin layer chromatography (ethyl acetate:hexane 3:2 $R_f=0.58$), with a melting point of 136°C. It was further hydrogenated as in Example 1 to remove selectively the

benzyl residue. The obtained P-1,3 cGP, compound **III**, was pure on thin layer chromatography (as above) with $R_f=0.15$ and melting point of 116°C .

Example 4: Synthesis of 1,3 cyclic propanediol phosphate (1,3 cPP)

1,3 cPP was prepared by reacting 1,3-propanediol (Aldrich) with an equimolar amount of phosphorus oxychloride and then purified as described by Buwalda et al., 1997. The product was isolated as the free acid (melting point: $99-100^{\circ}\text{C}$).

^{32}P labeled 1,3 cPP (1,3 cP ^{32}P) was prepared with $^{32}\text{POCl}_3$. The latter was obtained by introducing a trace of $\text{H}_3^{32}\text{PO}_4$ (Amersham) into an excess of POCl_3 in the cold (Neuhaus and Korkes, 1958). The reaction was then proceeded on a microscale and 1,3 cP ^{32}P was isolated by co-crystallization with unlabelled 1,3 cPP.

Example 5: Synthesis of 1,2 cyclic propanediol phosphate (1,2 cPP)

1,2 cPP was prepared by the same procedure as in Example 4 but using 1,2- propanediol (Aldrich). The compound was isolated as the Ba salt and was pure on paper chromatography (n-propanol:ammonia:water 6:3:1, $R_f=0.55$).

Example 6: Synthesis of phenyl 1,3 cyclic propanediol phosphate (P-1,3 cPP)

P-1,3 cPP was prepared by a method analogous to the procedure of Example 4, by reaction of 1,3-propanediol with an equimolar amount of phenyl phosphorodichloridate in dry pyridine. The product was crystallized twice from ethyl acetate-hexane and had a melting point of 72°C .

Example 7: Synthesis of phenyl 1,2 cyclic glycerophosphate (P-1,2 cGP)

This novel compound was prepared as in Example 3 by reaction of 1-benzyloxy-2,3-propanediol with phenyl- PO_2Cl_2 , followed by removal of the

benzyl residue by selective hydrogenation. Crystallization was achieved from ethanol-acetone and the product had a melting point of 95°C.

Example 8: Synthesis of phenyl 1,2 cyclic propanediol phosphate (P-1,2 cPP)

5

This novel compound was prepared as in Example 6 by reaction of 1,2-propanediol with an equimolar amount of phenyl-PO₂Cl₂ in dry pyridine. Crystallization was achieved from ethyl acetate-hexane and the product had a
10 melting point of 69°C.

Example 9: Synthesis of cyclic dihydroxyacetone phosphate (cdAP)

This novel compound was prepared by reaction of POCl₃ with dihydroxyacetone.

15 1.8 g (0.01M dimer or 0.02M monomer) Dihydroxyacetone dimer MW-180 dissolved in 20 ml fresh distilled methylene chloride.

3.07 g = 1.87 ml (0.02M) Phosphoryl Chloride (MW-153.5, d-1.645) in 4 ml MeCl₂ was slowly added to the solution at RT. The solution was refluxed for 15 h (the solution was black). Methylene chloride was evaporated and
20 100 ml 90% acetone/water was added to the solution. The reaction mixture was refluxed for 18 h. The black solution was treated with active carbon at RT and filtered. From the resulting slightly yellow solution was evaporated acetone and water and the very nice crystalline residue was dissolved in 10 ml acetone. 0.01 M BaJ₂ in 80 ml acetone was added to the solution and nice crystals of cyclic-
25 ~~dihydroxyacetone-phosphate barium salt started to precipitate. The precipitate~~
~~was washed 3 times with small quantities of acetone and dried. The product~~
~~was cleaned by dissolving it in small amounts of water and precipitating with~~
acetone. The resulting produce is white crystalline powder and shows in paper chromatography (solvents mixture: n-Propanol:NH₄H₂O 6:3:1) R_f - 0.50.

Example 10: Synthesis of phenyl cyclic dihydroxyacetone phosphate (P-dAP)

This novel compound was prepared by reaction of phenyl-PO₂Cl₂ with
5 dihydroxyacetone in dry pyridine. Upon removal of the solvent by vacuum, the
residue was extracted twice with ethyl acetate. After evaporation of the ethyl
acetate, an oily residue was obtained..

Example 11: Synthesis of cyclic oleyl lysophosphatidic acids (c-oLP)

10 These novel compounds were prepared by reaction of oleyl
lysophosphatidic acid (Avanti Polar Lipids) with excess
dicyclohexylcarbodiimide (DCC) in dimethyl sulfoxide. The product appeared
as a oil.

15 **BIOLOGICAL SECTION**

Materials and Methods

(i) Cells

Chinese hamster ovary (CHO) cells (Puck, 1985; Gottesman, 1987) and
NIH-3T3 cells (NIH), were used in the experiments. CHO cells were grown at
20 37°C in a humidified 5% CO₂ atmosphere in 60x15 mm Petri dishes (Falcon)
containing F12 medium supplemented with 10% fetal calf serum (FCS) and
2mM glutamine. When reaching near confluence, the cells were placed under
overnight starvation by replacing the medium to F12+2 mM glutamine with
0.1% FCS. The stimulation experiments were performed with cells which were
25 under starvation for 12-18 hours.

NIH-3T3 cells were grown under similar conditions in 1640 Eagle
medium at 10% bovine calf serum plus 2mM glutamine.

(ii) Tyrosine phosphorylation

The cyclic phosphates 1,3 cGP, 1,3 cPP, 1,2 cGP, 1,2 cPP, P-1,3 cGP and P-1,3 cPP and the controls α GP and β GP were dissolved in HBSS, or methanol to form stocks of 1mM or 10 mM. They were diluted into the cell culture medium to a final concentration of up to 20 μ M. At the assigned stimulation time (0.5-20 min.) the medium was aspirated under vacuum and the cells were washed 3 times with cold (4°C) phosphate buffered saline (PBS). The cells were then freeze-thawed 3 times with liquid nitrogen in 0.5ml of a lysis buffer of PBS containing 1mM phenylmethylsulfonyl fluoride, a cocktail of protease inhibitors (aprotinin, leuproptin, pepstatin A, phenanthroline, benzamidine HCl each at a concentration of 10 μ g/ml) and a cocktail of phosphatase inhibitors (80 mM β -glycerophosphate, 2 mM EGTA and 50 μ M Na₃VO₄), and then scraped off. The lysate was then vortexed and centrifuged for 2 minutes at 12.000 g. The supernatant was collected for further determinations by gel electrophoresis. The protein concentration in each sample, as evaluated by the Bradford assay, was in the range of 1-1.5 mg/ml. Aliquots of 10 μ g protein were applied onto 10% SDS-PAGE in a minigel set and resolved within 1 hour. The proteins were then transferred to nitrocellulose sheets for Western blotting. After blocking with a solution of 1% bovine serum albumine and 0.1% Tween 20 in PBS, the blots were incubated at 4°C for 16 hours with polyclonal rabbit anti-phosphotyrosine antibodies (Zymed Laboratories, San Francisco, CA) or monoclonal anti-phosphotyrosine antibodies (Transduction Laboratories, Lexington KY) and then washed several times. Bound antibodies were detected by horseradish peroxidase-conjugated anti-rabbit antibodies (Transduction Laboratories, Lexington KY) after 2 hours incubation at room temperature, using the conventional ECL detection method. Control assays were carried out in the presence of 5mM phosphotyrosine or without the phosphotyrosine antibodies. In both, the intensity of the bands was reduced to background level.

(iii) Threonine phosphorylation

Blots were tested analogously for threonine phosphorylation using polyclonal rabbit anti-phosphothreonine antibodies (Zymed Laboratories, San Francisco, CA).

5

(iv) In-gel kinase assay

Phosphorylated cell proteins were separated by SDS-PAGE using a gel that was copolymerized with MBP (0.5mg/ml) and treated as described (Karunuguran *et al.*, *EMBO J.* 15:254-264, 1996). After electrophoresis, the gel
10 was fixed with isopropanol and denatured using 6M Urea. Renaturation was achieved by gradual removal of the excess of urea followed by extensive washings in a renaturing buffer (16 h, 4°C) and in a buffer containing 20mM HEPES pH 7.6, and 20 mM MgCl₂ (30 min, 30°C). The gel was then subjected to phosphorylation in a buffer containing 20mM HEPES pH 7.6, 20 mM
15 MgCl₂, 2mM DTT, 20 μM ATP and 100 mCi [γ ³²P]-ATP (30°C for 120 min). Finally the gel was extensively washed, dried, and subjected to autoradiography.

(v) Immunoprecipitation kinase assays. Raf-1 activity was determined by
20 immunoprecipitation with anti-Raf-1 C terminus antibodies (Santa Cruz Biotech, CA) using recombinant MEK1 as a substrate as previously described (Seger, 1994). Mitogen-activated protein kinase (MAPK) activity was determined by immunoprecipitation with anti-ERK2 C terminus antibodies (Santa Cruz Biotech, CA) using MBP as a substrate (Seger, 1994). Jun
25 N-terminal kinase (JNK) activity was determined by purifying the JNK on a GST-Jun (1-97) column followed by phosphorylation (Hibi, 1993).

RESULTS

Example 12

CHO cells were contacted for 1 min. at 37°C with 1 μ M of α GP, β GP, 1,3cGP(E), P-1,3-cPP and the level of tyrosine phosphorylated proteins in the
5 cells was then determined by using polyclonal anti phosphotyrosine antibodies as explained above.

As seen in Fig. 1, augmented tyrosine phosphorylation was induced by all of the above cyclic phosphates in a series of proteins as seen in several major bands having μ M of about 35 kD, 45 kD, 60 - 70 kD and about 120 kD.

10

Example 13

CHO cells were contacted for 1 min. with the same linear and cyclic glycerophosphates described in Example 12 above under the same conditions. Determination of the level of tyrosine phosphorylated proteins in the cells was
15 determined using monoclonal anti phosphotyrosine antibodies as described above. Using this antibody, the detected tyrosine phosphorylation was in bands having a molecular weight of about 35 kD and of about 45 kD (results not shown).

20 Example 14

CHO cells were incubated for a period of 1 min., 3 mins., 5 mins. or 10 mins. at 37°C with 1 μ M or 2 μ M of 1,3-cPP. The level of tyrosine phosphorylated proteins in the cells was determined using monoclonal anti phosphotyrosine antibodies.

25

As seen in Fig. 2, phosphorylation was most markedly seen in the band(s) having a molecular weight of about 35 kD and about 45 kD.

Example 15

The level of tyrosine phosphorylated proteins in NIH 3T3 cells after their incubation for 1 min. at 37°C with α GP, β GP, 1,3cGP(E), P-1-3 cPP. The level of tyrosine phosphorylated proteins in the cells was determined using polyclonal anti phosphotyrosine antibodies.

Augmented tyrosine phosphorylation was induced in the cells by all the above cyclic phosphates (results not shown).

Example 16

The level of tyrosine phosphorylated proteins in NIH 3T3 cells was determined as described above after their incubation for either 1 min. or 5 mins. at 37°C with 0.5 μ M, 1 μ M, 2 μ M or 4 μ M of 1,3-cPP. The level of tyrosine phosphorylated proteins was determined using monoclonal anti phosphotyrosine antibodies as described above.

As seen in Fig. 3, augmented tyrosine phosphorylation was seen in the cells similar to that seen in the cells of Example 14 above.

Example 17

Induction of tyrosine phosphorylation in cells by 5-membered ring cyclic phosphate (see Appendix 1) was carried out as described in Example 12 above.

The pattern of tyrosine phosphorylation was similar to that obtained by incubation of the cells with the 6-membered ring cyclic phosphates but at a relatively higher concentration and at longer incubation times (results not shown).

Example 18

~~The kinetics of tyrosine phosphorylation in proteins of CHO cells~~
incubated with either 5 μ M or 10 μ M of 1,2-cPP at 37°C for a period of 1 min., 3 mins. or 5 mins. was determined as described above using monoclonal anti phosphotyrosine antibodies. As seen in Fig. 4, enhanced tyrosine

phosphorylation was detected at bands having μ M of about 35 kD, 60 – 70 kD, 120 kD and an additional band of about 38 kD. Similar results were obtained using the deoxy analog of 1,2-cPP (results not shown).

5 **Example 19**

To further verify the induced tyrosine phosphorylation shown in the above examples, *in situ* ^{32}P labeling of CHO cells was carried out under a pulse of cyclic phosphate as described above. 10^7 CHO cells/per 1 ml medium were pulsed with 1 mCi of $^{32}\text{PO}_4^{3-}$ for 12 hours and then activated for 2 mins. with 2
10 μM of 1,3-cGP(E) or 2 μM of 1,3-cPP. As control, the same cells following the 12 hour pulsing were not contacted with the cyclic cGPs. Massive ^{32}P protein labeling was observed in both the cells which were incubated with the cyclic GMPs as well as with one set that were not.

However, as seen in Fig. 5, following treatment of the gel with 1M of
15 KOH for 2½ hours at 25°C or 37°C (which selectively hydrolyzes the phosphoserine and phosphothreonine residues (Kozma *et al.*, *J. Methods Enz.*, 201:28-43, 1991), two major ^{32}P bands of phosphorylated tyrosine at molecular weights of about 35 kD and 45 kD immersed in the treated samples. These bands could be correlated with kinase activity (see Example 20 below).

20

Example 20

The in-gel kinase assay in the presence of MBP was applied as described in Materials and Methods above to detect stimulation of protein kinases by the cyclic phosphates. CHO cells were treated either with medium (0 control), or
25 with 2 μM , α GP or β GP for 2 minutes, 2 μM 1,3-cGP (E) for 2 and 5 minutes and 2 M 1,3-cPP for 2 minutes after which the cells were lysed and analyzed.

As seen in Fig. 6, at least three MBP phosphorylated kinases could be detected by this method. Their apparent molecular weights were around MW of 35-40, 55 and 62 kD. The 35-40 kD protein kinase which was activated by

1,3-cGP (E) could be assigned to the P42 MAPK (ERK 2). In addition, cells were treated as described above and then subjected to Raf-1, MAPK and IP kinase assays and these are presented in the first and second rows of Fig. 6. In parallel, JNK assay was performed (third row, JST-Jun substrate). The results
5 strongly suggest that the Raf1/MAPK cascade is activated up to approximately 5 fold by 1,3-cGP (E), whereas the JNK pathway seems to be unaffected.

Example 21

In both the CHO and the NIH-3T3 cells a high level of constitutive
10 phosphoserine containing proteins did not permit an unequivocal detection of changes induced by the tested cyclic phosphates. However, as seen in Fig. 7, a marked augmentation in threonine phosphorylation induced by the six membered ring cyclic phosphates could be clearly detected in three specific proteins (18 kD, 35 kD, 38 kD). As shown, at 25°C, the bands at molecular
15 weight of about 35 kD and about 38 kD reached a maximum phosphorylation at around 7 mins. which was slowly diminished while the band at about 18 kD displayed a much sharper phosphorylation-dephosphorylation profile. The presence of 5 mM phosphothreonine in the antibody binding assay reduced the intensity of the 35 KD band to the control level.

20

Example 22

In principle, cyclic phosphates can be hydrolyzed to the respective linear forms (e.g. α -GP) either spontaneously or through putative phosphodiesterases. These possibilities were tested with the 4 cyclic phosphates (1,2 cGP, 1,3 cGP,
25 1,2 cPP and 1,3 cPPP (see Appendix 1) in either aqueous solution or in cell lysates of CHO or NIH-3T3 cells (see Materials and Methods). 10 mg/ml cyclic phosphate in 1 ml of either PBS or PBS mixed with cell lysate (0.1 mg/ml protein) and incubated at 37° for up to 24 hours. Samples were tested at different times by thin layer chromatography on Silica gel 60 with n-propanol,

concentrated ammonia, H₂O (6:3:1 v/v/v). The R_f values for all 4 cyclic phosphates was in the range of 0.45-0.55 while α -GP and β -GP had R_f =0.14-0.16. This distinct difference allowed the qualitative detection of hydrolysis of the cyclic phosphate. No hydrolysis of the tested cyclic phosphates either in PBS or by the cell lysate was detected. Furthermore, aqueous solutions of all the 6 cyclic phosphates were found to be stable for at least several days at room temperature and for months below 0°C.

Example 23

Inhibition of phosphatase activity of cell lysate by cyclic phosphates was assayed in 1.0 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM P-nitrophenyl phosphate, PNPP, as a substrate. Reaction was initiated by the addition of crude cell lysate (100 μ g/ml final protein concentration) and terminated after 90 minutes at 37°C by an addition of 50 μ l of 1M NaOH. The absorbance of the released P-nitrophenol was measured at 405 nm. Assays were conducted under the same conditions as above in the presence of 50 μ M cyclic phosphate. The enzyme activity in the absence of inhibitor was taken as 100% activity.

Only 1,3 cGP(E) obtained from PLase C cleavage of PG (see Materials and Methods) displayed a limited inhibition of the lysate phosphatases.

Synthetic 1,3 cGP, unlike the enzymatic product displayed only approximately 10% inhibition at 50 μ M concentration. Similarly, 50 μ M 1,3 cPP displayed 15-20% inhibitory capacity. All other cyclic phosphates, as well as α -GP and β -GP at a concentration of 50 μ M induced less than 5% inhibition of the cell lysate phosphatase activity.

~~In a series of analogous experiments phosphodiesterase activity of the~~
cell lysates in the presence and absence of the cyclic phosphates were tested. In these experiments bis P-nitrophenyl phosphodiester was used as a substrate. No

significant effect of any of the cyclic phosphates on the cell lysate phosphodiesterase activity was observed (not shown).

Example 24

5 Binding and incorporation assays were carried out with ^{32}P labeled 1,3-cPP (1,3-cP ^{32}P), the only one of the cyclic phosphates which could be synthesized in a relatively high specific activity (see Materials and Methods). When contact with CHO cells there was a rapid apparent binding of 1,3-cP ^{32}P followed by a continuous uptake which leveled off after approximately 2 hours
10 of incubation. A typical profile of binding and incorporation of 1,3-cP ^{32}P is shown in Figure 8. In this experiment a triplicate of 1 ml samples each containing 2×10^6 CHO cells and $10 \mu\text{M}$ 1,3-cP ^{32}P (approximate specific activity $10 \mu\text{Ci}$ per μmole) were incubated in the starvation medium for different times. After 2 washings with PBS the cells were disintegrated in 1 M
15 NaOH and radioactivity was scored with a β counter. The insert presents the magnified uptake profile for short incubations (up to 2 minutes) where the intercept (0 time incubation) corresponded to approximately 3×10^6 molecules per cell, while at equilibrium (above 2 hours incubation) incorporation corresponded to approximately 2×10^8 molecules per cell. This profile remained
20 essentially unaltered in competition experiments where 1,3-cP ^{32}P was mixed with increasing amounts of unlabeled 1,3-cPP or 1,3-cGP, indicating that the apparent binding extrapolated for 0 time incubation and the following incorporation proceeded through non-specific binding.

~~Without being limited to theory this mode of uptake may be explained by~~
25 a mechanism by which 1,3-cPP and probably the other cyclic phosphates as well, first incorporate rapidly and non specifically into the cell plasma membrane (probably the lipid layer), where it reaches a steady-state concentration after a few seconds. Through this compartment the cyclic phosphate partitions further with the cytosol to reach an equilibrium with the

external medium after approximately 2 hours. This passive partitioning mechanism is further supported by the estimated concentration of the intracellular concentration of 1,3 cP³²P at the equilibrium which is in the micromolar range, i.e. the range of the external 1,3 cP³²P concentration. It is of interest to note that after 5 minutes of incubation, when tyrosine phosphorylation is maximal (see for example Figure 3) the intracellular concentration of the cyclic phosphate is in the order of 10⁷ - 10⁸ molecules per cell.

10 Example 25

In an attempt to characterize the intracellular binding of the cyclic phosphates, perforated CHO cells (2x10⁶ in 1 ml of 1:1 v/v ethanol in PBS) were incubated for 5 minutes at 4°C with 20 μM 1,3-cP³²P (see above) and increasing amounts of either αGP, unlabeled 1,3-cPP or 1,3-cGP. Radioactivity was scored after 2 washings with PBS at 4°C as above. As shown in Figure 9, competition with unlabeled 1,3-cPP is displayed indicating specific binding. Similarly, clear binding competition was also observed with 1,3-cGP while αGP was ineffective. In this set of experiments the number of displaceable (i.e. specifically bound) ligands was estimated to be in the range of 10⁷ - 10⁸ per cell. Such a high number suggests low affinity ubiquitous targets, rather than specific receptors.

Example 26

The effect of the cyclic phosphates on the differentiation of human breast cancer cells was determined by detecting cell marker characteristics connected with differentiation. Highly undifferentiated breast cancer cells are characterized by low level of progesterone and estrogen receptors. Patients with such tumor have a poor prognosis. Partially differentiated breast cancer cells contain a significantly higher level of these receptors. Two available cell lines

(established by Prof. Y. Kedar, Tel Aviv University, Tel Aviv, Israel) are used, which are of low and high estrogen and progesterone receptors, respectively. Cells are incubated in tissue culture medium with or without 1-10 μ M cyclic phosphate or α GP (as a control). Change in receptor level in these cells is
5 monitored by conventional Western Blot analysis using known anti estrogen and anti progesterone receptor monoclonal antibodies.

Example 27

High virulent human T-leukemia cell lines (e.g. Jurkat cells) are cultured
10 with various cyclic phosphates as above. The effect of the cyclic phosphates in these cells is monitored by the emergence of differentiation markers on the cell surface (e.g. CD3). Their quantity is determined by conventional FACS analysis using fluorescent antibodies.

15 Example 28

Binding of insulin to its receptor on the cell surface results in rapid tyrosine phosphorylation on the 117 kD and 200 kD receptor proteins. This is the initial event in the overt functions of insulin (e.g. glucose uptake).

CHO-T cells expressing the insulin receptor (obtained from Prof. Y.
20 Zick, The Weizmann Institute, Rehovot, Israel) were divided into four groups, each incubated for 20 mins. with one of the following:

Group 1: 1 nM insulin which results in maximal phosphorylation of the receptors.

Group 2: ~~0.1 nM insulin resulting in partial phosphorylation of the~~
25 ~~receptors, in situation analogous to refractory response~~
~~such as occurs in IDDM Type 2.~~

Group 3: 0.1 nM insulin and 0.1 μ M 1,3, cPP; and

Group 4: 0.1 μ M 1,3, cPP only.

Tyrosine phosphorylation was determined using anti-phosphotyrosine antibody (as described above). Phosphorylation was determined in the two protein bands of the insulin receptor having a molecular weight of 117 kD (Fig. 10A) and 200 kD (Fig. 10B).

5 As seen in Fig. 10, incubation of the insulin receptor expressing cells with a combination of insulin (0.1 nM) and 1,3 cPP almost doubled the phosphorylation obtained in the cells incubated with insulin only. Thus, the tested GC shows synergistic activity with insulin.

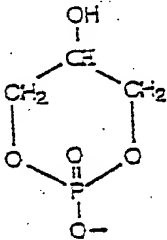
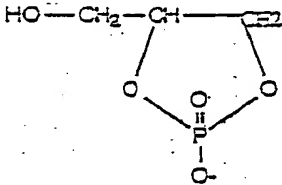
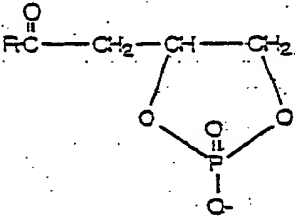
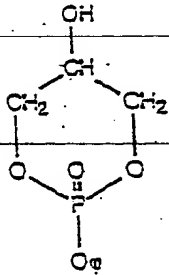
10 **Example 29**

CHO cells expressing the human growth factor receptor (HGF) Erb-b2 (prepared by Y. Yarden, Weizmann Institute, Rehovot, Israel) are divided into the following four groups:

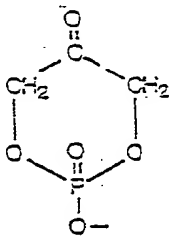
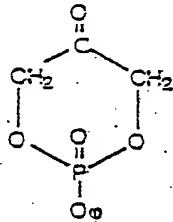
- 15 Group 1: Cells incubated for about 20 mins. with HGF at a concentration resulting in maximal phosphorylation of the HGF receptor;
- Group 2: Cells incubated with concentrations of HGF resulting in partial phosphorylation of the receptor;
- Group 3: Cells incubated with a combination of HGF in the lower concentration and the tested cyclic glycerophosphate; and
- 20 Group 4: Cells incubated with the tested glycerophosphate alone.

The percent of tyrosine phosphorylation of Erb B2 is monitored using known antibodies as described above.

Appendix A

	Formula	Abbreviation
I		1,3 cGP
II		1,2 cGP
III		cyclic lysophosphatidic acid, c-lysoPA
IV		P-1,3 cGP

	Formula	Abbreviation
V		P-1,2 cGP
VI		1,3 cPP
VII		1,2 cPP
VIII		P-1,3 cPP
IX		P-1,2, cPP

	Formula	Abbreviation
X		cDHAP
XI		P-cDHAP

- 



-

6. A pharmaceutical composition according to any one of Claims 1-4, wherein m is 0.
7. A pharmaceutical composition according to Claim 6, wherein X is C₁ - C₂₄ alkyl.
- 5 8. A pharmaceutical composition according to Claim 7, wherein X is methyl.
9. A pharmaceutical composition according to Claim 8, comprising 1,2-cyclic propanediol phosphate.
- 10 10. A pharmaceutical composition according to Claim 8, comprising phenyl 1,2-cyclic propanediol phosphate.
11. A pharmaceutical composition according to Claim 6, wherein X is -CH₂OH.
12. A pharmaceutical composition according to Claim 11, comprising 1,2-cyclic glycerophosphate.
- 15 13. A pharmaceutical composition according to Claim 11, comprising phenyl 1,2-cyclic glycerophosphate.
14. A pharmaceutical composition according to Claim 6, wherein X is -CH₂acyl.
- 15 15. A pharmaceutical composition according to Claim 14, comprising 3-acyl 1,2-cyclic glycerophosphate.
- 20 16. A pharmaceutical composition according to Claim 15, comprising cyclic oleyl lysophosphatidic acid.
17. A pharmaceutical composition according to any one of Claims 1-4, wherein Y is -CH₂-.
-
- 25 18. A pharmaceutical composition according to Claim 17, comprising 1,3-cyclic propanediol phosphate.
-
19. A pharmaceutical composition according to Claim 17, comprising phenyl 1,3-cyclic propanediol phosphate.

20. A pharmaceutical composition according to any one of Claims 1-4, wherein Y is - CH(OH) -.
21. A pharmaceutical composition according to Claim 20, comprising 1,3-cyclic glycerophosphate.
- 5 22. A pharmaceutical composition according to Claim 20, comprising phenyl 1,3-cyclic glycerophosphate.
23. A pharmaceutical composition according to any one of Claims 1-4, wherein Y is - C(=O) -.
24. A pharmaceutical composition according to Claim 23, comprising cyclic
10 dihydroxyacetone phosphate.
25. A pharmaceutical composition according to Claim 23, comprising phenyl cyclic dihydroxyacetone phosphate.
26. A pharmaceutical composition for inducing phosphorylation in intracellular proteins of target cells comprising a pharmaceutically acceptable
15 carrier and, as an active material, a compound of general Formula I of Claim 1.
27. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and, as an active material, a compound of the general Formula I of Claim 1 for promotion of cell differentiation in target cells.
28. A pharmaceutical composition according to Claim 27, for the treatment
20 of malignant diseases and disorders.
29. A pharmaceutical composition according to Claim 28, wherein said malignant disorder is a blood malignancy.
30. A pharmaceutical composition according to Claim 29, wherein said
~~blood malignancy is leukemia.~~
-
- 25 31. A pharmaceutical composition according to Claim 28, wherein said
~~malignancy is breast cancer.~~
-
32. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and, as an active material, a compound of the general Formula 1 of Claim 1, for induction of hormone-like signaling.

33. A pharmaceutical composition according to Claim 32, wherein said hormone is insulin and the composition is for the treatment of non-insulin-dependent diabetes mellitus (non-IDDM type II diabetes).
34. A pharmaceutical composition according to Claim 32, wherein said hormone is human growth hormone (HGH) for the treatment of disorders in which HGH is involved.
35. A pharmaceutical composition according to Claim 32, wherein said hormone is epidermal growth factor (EGF) for the treatment of disorders involving EGF.
36. A compound of the general Formula I of Claim 1, with the exception of the following compounds:
- i. compounds wherein Y is $-(CH_2)_m-$, m is 0, X is CH_3 , $-CH_2OH$ or CH_2Oacyl wherein acyl is a saturated carboxylic acyl with more than 12 carbon atoms, and R is H or a cation;
 - ii. compounds wherein Y is $-(CH_2)_m-$, m is 1, X is H and R is H, a cation or phenyl; and
 - iii. compounds wherein Y is $-CH(OH)-$, X is H and R is H, a cation or phenyl.
37. A compound according to Claim 36, selected from the group consisting of:
- i. phenyl 1,2 cyclic glycerophosphate;
 - ii. phenyl 1,2 cyclic propanediol phosphate;
 - iii. cyclic dihydroxyacetone phosphate;
 - iv. phenyl cyclic dihydroxyacetone phosphate; and
 - v. cyclic oleyl lysophosphatidic acid.
- ~~38. A method for treatment of disorders and diseases which can be treated~~
by phosphorylation of internal proteins comprising administering to the individual in need a therapeutically effective amount of a compound of general Formula I of Claim 1.

39. A method for the treatment of malignant diseases comprising administering to an individual in need a therapeutically effective amount of the compound of Formula I of Claim 1.

40. A method according to Claim 39, wherein said malignant disease or disorder is blood malignancy.

41. A method according to Claim 40, wherein said blood malignancy is leukemia.

42. A method according to Claim 39, wherein said malignant disease is breast cancer.

43. A method for the treatment of diseases involving hormone-like signaling comprising administering to an individual in need a therapeutically effective amount of the compound of Formula I of Claim 1.

44. A method according to Claim 43, wherein said hormone is insulin for the treatment of non-IDDM type II diabetes.

45. A method according to Claim 43, wherein said hormone is human growth hormone (HGH) for the treatment of disorders in which HGH is involved.

46. A method according to Claim 43, wherein said hormone is epidermal growth factor (EGF) for the treatment of disorders involving EGF.

47. A method for detecting abnormal conditions of a tested cell comprising:

- i. contacting the cells with cyclic glycerophosphates or their analogs;
- ii. detecting the level of phosphorylation in intracellular proteins of the tested cells; and
- iii. comparing said level of phosphorylation to the level of phosphorylation in intracellular proteins of normal cells following contact with said cGP, a level of phosphorylation differing from that detected in the normal cells indicating a high probability of abnormality in the tested cells.

48. Use of a compound of the general Formula I as defined in Claim 1, for preparation of a medicament for the treatment of disorders and diseases that can be treated by phosphorylation of intracellular protein, substantially as described in the specification.

5 49. Use of a compound of the general Formula I as defined in Claim 1, for the preparation of a medicament for the treatment of malignant diseases and disorders, substantially as described in the specification.

50. Use of a compound of the general Formula I as defined in Claim 1, for the preparation of a medicament for the treatment of diseases or disorders
10 involving hormone-like signaling, substantially as described in the specification.

51. Use of a compound according to Claim 36, in the preparation of a medicament, substantially as described in the specification.

52. Use of a compound according to Claim 37, in the preparation of a
15 medicament, substantially as described in the specification.

For the Applicants,
REINHOLD COHN AND PARTNERS

By: 

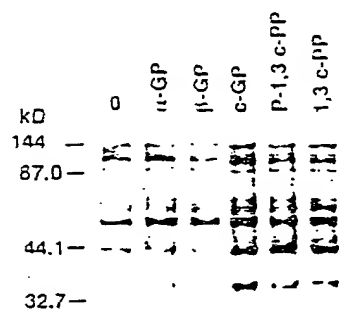


Fig. 1

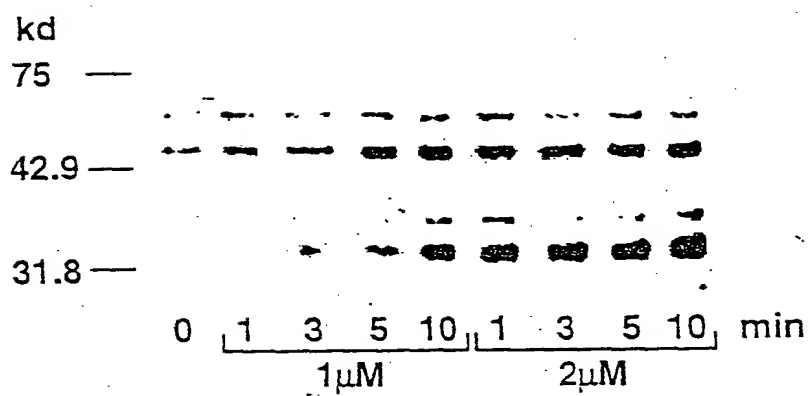


Fig. 2

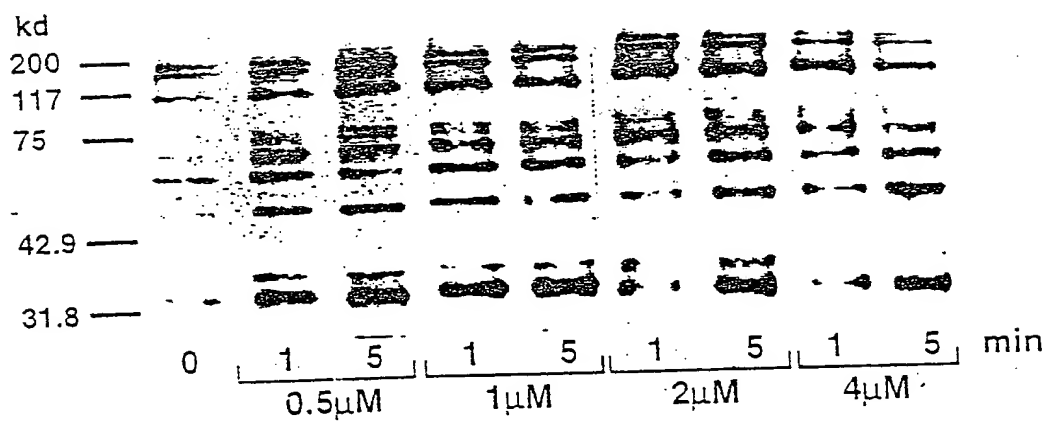


Fig. 3

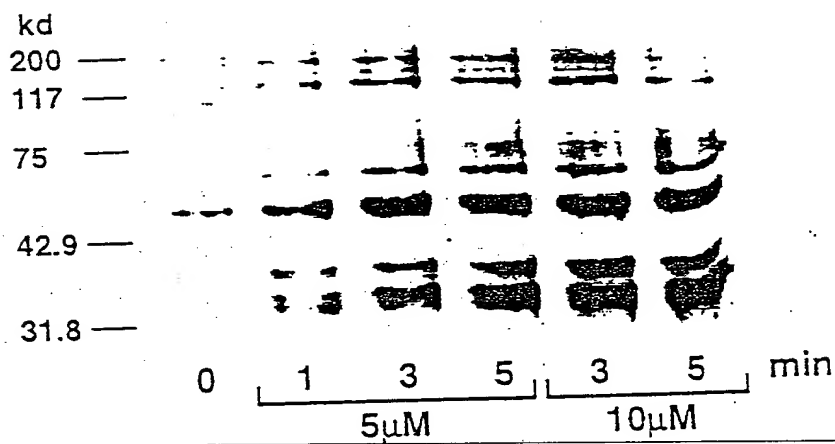


Fig. 4

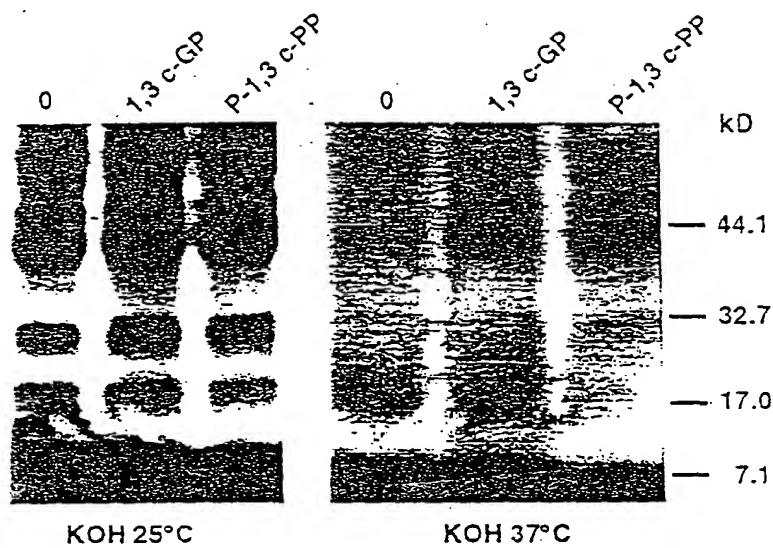


Fig. 5

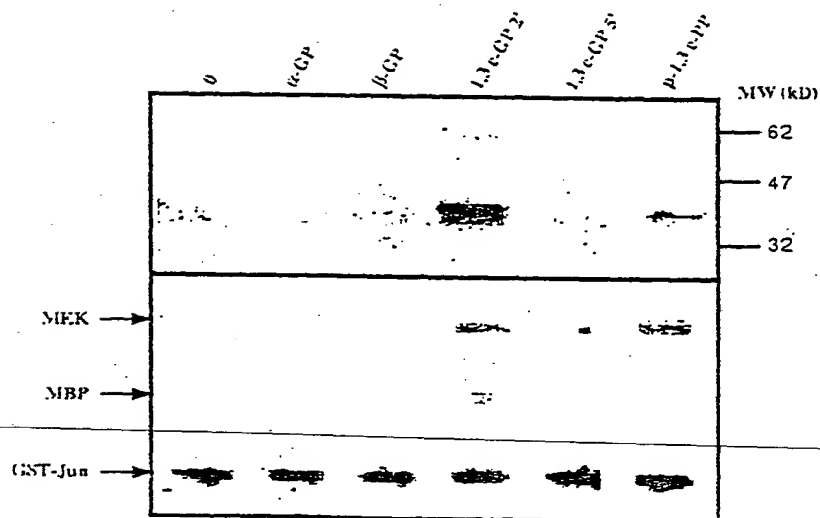


Fig. 6

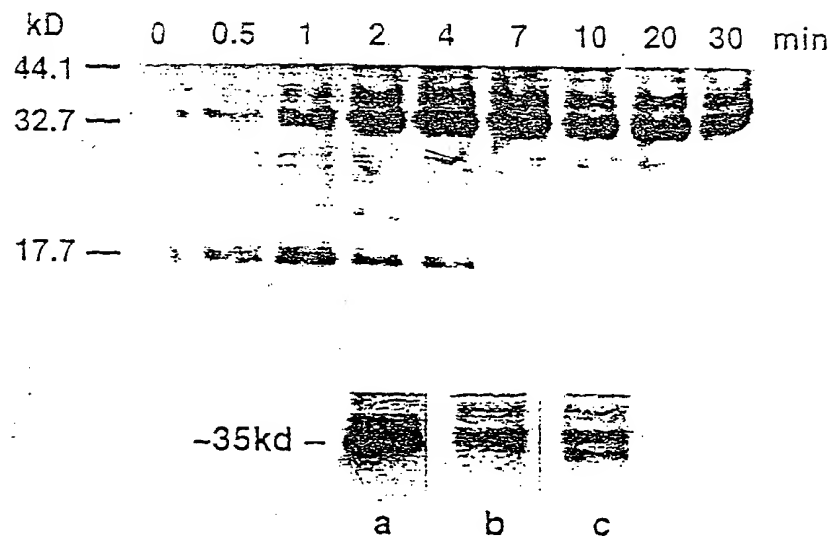


Fig. 7

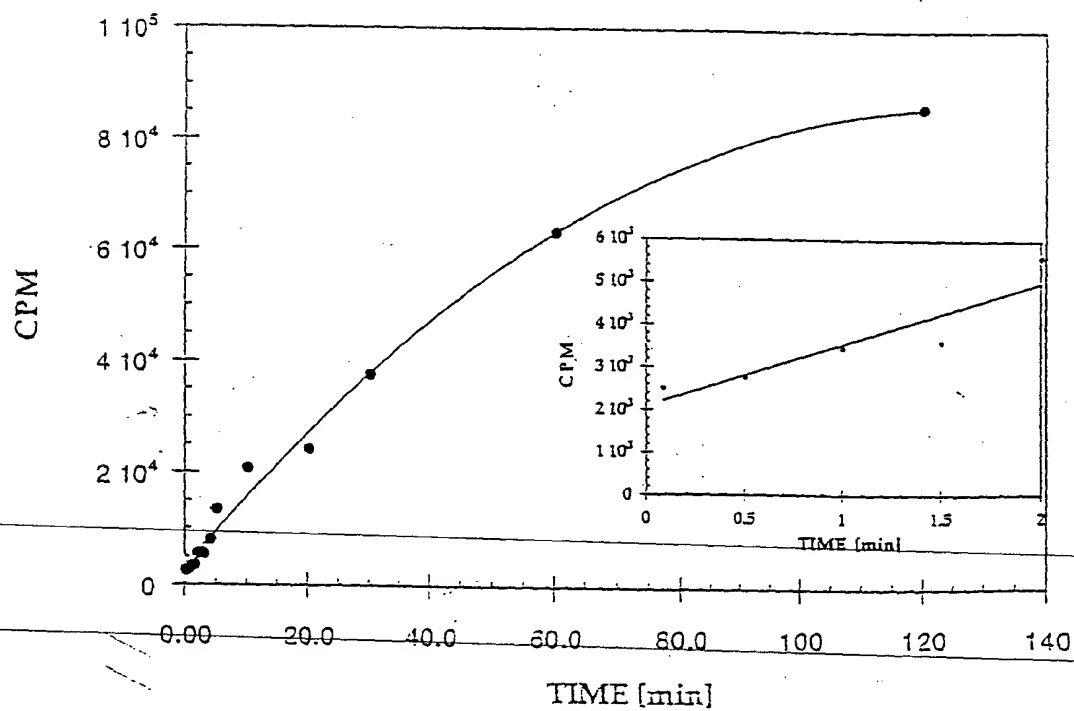


Fig. 8

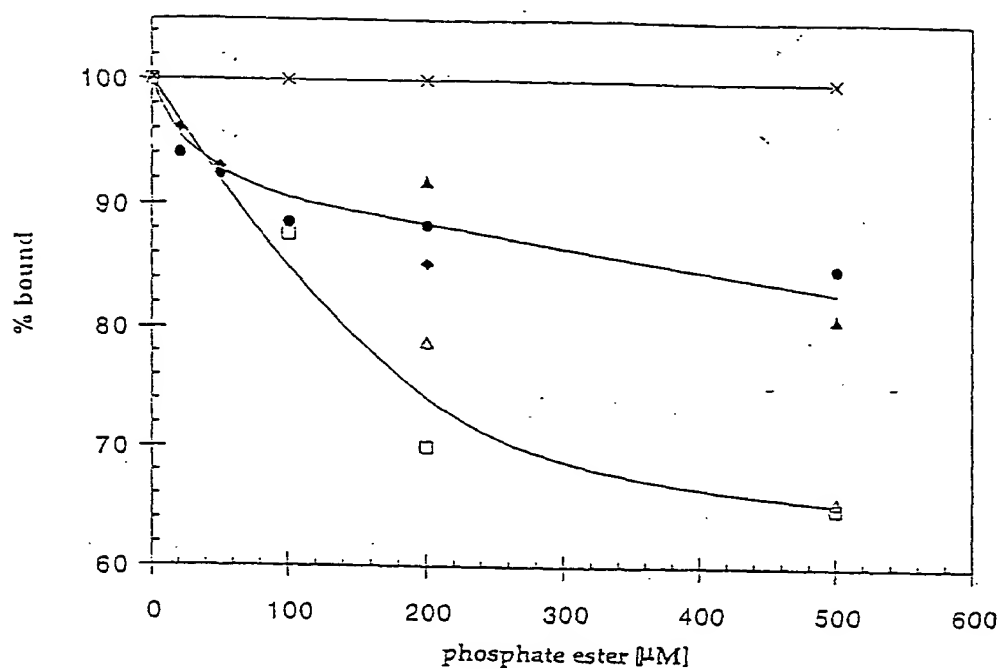


Fig. 9

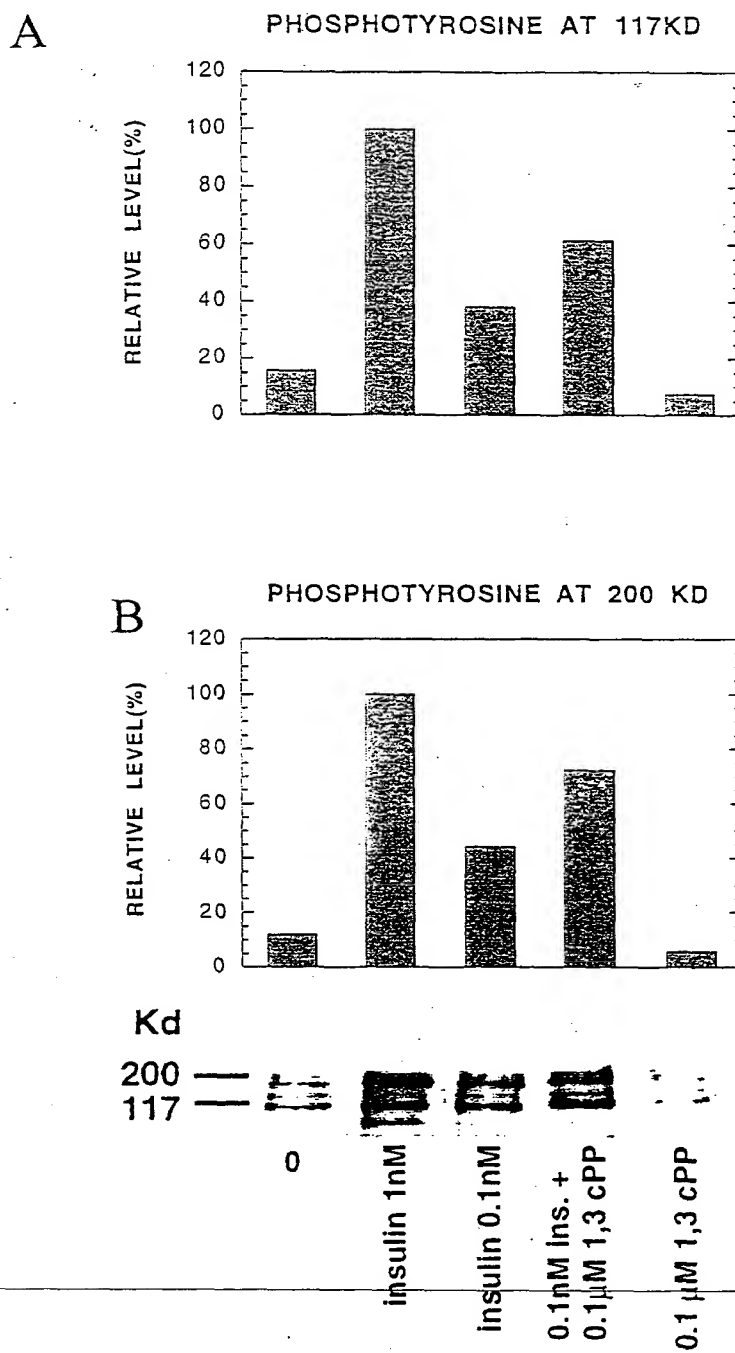


Fig. 10

